# Expressions of HBV X gene regulated by different promoters and their effects on cell apoptosis

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**Abstract.** – **OBJECTIVE:** To investigate the difference in expression level of hepatitis B virus (HBV) X gene, and to further study the difference of HBV X protein (HBx) at varied expression levels in apoptosis regulation of hepatocellular carcinoma cells. The recombinant plasmid rPX with HBV enhancer 1 (Enh1), X gene promoter, X gene and polyA tail were constructed, respectively.

**PATIENTS AND METHODS:** HepG2 cells were transiently transfected with the recombinant plasmids and control vector pGEM®-7Zf (+) by virtue of Fugene®HD; reverse transcription PCR (RT-PCR) and Western blotting were applied to analyze the transcription and expression of HBV X gene as well as the difference in the expression level in multiple groups. The activity of the transfected cells in each group was detected by using the methyl thiazolyl tetrazolium (MTT) method for 6 consecutive days after transfection. A flow cytometer was utilized to measure the cell apoptosis rate.

**RESULTS:** The RT-PCR results showed that messenger RNA expression of HBV X gene was detected in all HepG2 cells transfected by different recombinant plasmids, of which the relationships of the expression levels were rCX->rEX1 and rEX2> rPX ($p$<0.05). Only HepG2/rCX cells in each group of transfected cells showed HBx expression by Western blot. MTT method revealed that there were notable differences between HepG2/rCX, HepG2/rEX1, HepG2/rPX and HepG2/pGEM®-7Zf (+) ($p$<0.05). The apoptosis rates of HepG2/rCX, HepG2/rEX1 and HepG2/rPX were significantly higher than that of HepG2/pGEM®-7Zf (+) ($p$<0.05).

**CONCLUSIONS:** HBx can promote cell apoptosis. Results of this research also indicate that there is a significant difference in the pro-apoptotic role of HBx when its expression is regulated by different promoters, and such a difference may be a part of the complex pathogenic mechanisms of HBV and hepatocellular carcinoma.

## Introduction

Hepatitis B virus (HBV) infection is widespread around the world, and as one of the high-risk infectious diseases influencing people's health, it can increase the incidence rate of hepatocellular carcinoma[1-3]. The disorder of cell apoptosis plays an important role in the occurrence of hepatocellular carcinoma, and current researches[4-6] have found that HBV X protein (HBx) has a close association with the disease; therefore, it is necessary to provide an experimental basis for the pathogenesis of hepatocellular carcinoma, especially the HBx and cell apoptosis, so as to further investigate the relationship between HBx and hepatocellular carcinoma. In this research, by constructing recombinant plasmids in which the expression of HBV X gene was regulated by different promoters, the differences in the expression levels of HBV X gene were observed, and the differences in the regulatory effects of HBx at varied expression levels on apoptosis of hepatocellular carcinoma cell HepG2 were further investigated, thus providing a theoretical basis for elaborating the mechanism of HBx in triggering hepatocellular carcinoma.

## Materials and Methods

### Vectors, Strains and Cells

Cloning vector pGEM®-7Zf (+) was purchased from Promega Corporation, (Madison, WI, USA),

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and vectors payw1.2 and payw1.2m which contained 1.2 copies of the wild-type HBV genome were stored in this laboratory. *Escherichia coli* JM109 was purchased from TaKaRa (Otsu, Shiga, Japan) PEGFP-C1 plasmid and HepG2 cells were preserved in this laboratory. The study was approved by the Ethics Committee of Xiangyang No.1 People's Hospital, Hubei University of Medicine.

### Major Reagents

Taq DNA polymerase, restriction enzyme (HindIII, KpnI, NcoI, ApaI, BamIII, SphI and BspHI), T4 DNA ligase and DNA Marker (2000 bp and 15000 bp) were purchased from TaKaRa (Otsu, Shiga, Japan) Co., Ltd. E.Z.N.A.® Gel Extraction kit was bought from Omega Bio-Tek, (Norcross, GA, USA). The plasmid extraction kit was purchased from Qiagen (Hilden, Germany). The transfection reagent fuGENE® HD was bought from Roche (Basel, Switzerland), and TRIzol kit from Invitrogen (Carlsbad, CA, USA). Rabbit anti-human HBx polyclonal antibody, rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody and secondary goat-anti-rabbit antibody [horseradish peroxidase (HRP)-labeled] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The measurement kit of apoptosis flow cytometer was bought from eBioscience (San Diego, CA, USA).

### Construction of Recombinant Plasmids in which the Expressions of HBV X Gene Were Regulated by Different Promoters

The cytomegalovirus (CMV) promoter, HBV X gene and polyadenylic acid (polyA) were connected and then inserted into pGEM®-7Zf(+) Vector to construct the recombinant plasmid rCX; plasmid payw1.2 which contained 1.2 copies of the wild-type HBV genome and payw1.2 in which the mutation of the eighth codon of corresponding X gene was terminated were used as the templates; polymerase chain reaction (PCR) was used to amplify HBV enhancer 1 (Enh1), X gene promoter, X gene and polyA tail which were inserted into pGEM®-7Zf(+) Vector to construct the recombinant plasmids rEX1 and rEX2. Double-enzyme digestion method was applied to isolate the fragments containing the X gene promoter, X gene and polyA tail from payw1.2, and they were inserted into pGEM®-7Zf(+) Vector to construct the recombinant plasmid rPX. Colony PCR and sequencing were adopted for identification.

### Cell Transfection

2 µg recombinant plasmids rCX, rEX1, rEX2 and rPX, as well as pGEM®-7Zf (+) Vector, were taken respectively to transfect the HepG2 cells in accordance with the kit instructions. The method is as follows: OPTIMEM culture medium and fuGENE®HD was kept at room temperature before being used. 2 µg plasmid were mixed with 100 µl OPTIMEM, followed by the addition of 8 µl fuGENE®HD. After keeping at room temperature for 15 minutes, transfection mixture was gently mixed with the cells. After that, cells were cultured in 6-well plate at 37°C in an incubator.

### Detection of Gene Transcription Via Reverse Transcription PCR (RT-PCR)

The cells and HepG2 transfected by the 5 kinds of plasmids were cultured for another 48 hours; TRIzol reagent was used to extract the total RNA in the cells, and RT-PCR was conducted to detect the X gene expressions of different plasmids. The primer sequences are shown in Table I.

### Detection of HBx Expressions

8 µg recombinant plasmids rCX, rEX1, rEX2 and rPX, as well as pGEM®-7Zf (+) Vector, were taken respectively to transfect the HepG2 cells; the total proteins of the cells were extracted 48 hours after transfection, and Western blotting was performed to measure the HBx expressions in the transfection groups.

### Detection of Cell Growth Conditions in Transfection Groups via Methyl Thiazolyl Tetrazolium (MTT)

HepG2/rCX, HepG2/rEX1, HepG2/rEX2, HepG2/rPX, HepG2/pGEM®-7Zf (+) on day 2 after transfection and control HepG2 cells were seeded into a 96-well plate at $1\times10^3$ cells/well, respectively; 10 µL MTT (5 mg/mL) were added to each well in 1-6 days later, and further cultured at 37°C for 4 h. After the culture was terminated, the supernatant in the wells was absorbed and discarded carefully, and 150 µL dimethyl sulfoxide (DMSO) were added to each well and shaken for 10 minutes, followed by measurement of optical density at the wavelength of 490 nm ($OD_{490}$).

### Detection of Cell Apoptosis

The transfected cells and the control HepG2 cells were taken 48 hours after transfection for Annexin V/propidium iodide (PI) staining, and the flow cytometer was utilized to detect the apoptosis rates of the cells.
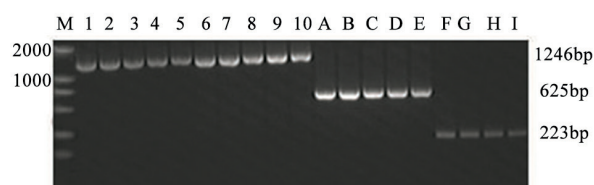
**Figure 1. Identification of recombinant plasmids via colony PCR.** M: marker; 1-5: Colony PCR for rEX1; 6-10: colony PCR for rEX2; A-E: colony PCR for rCX; F-I: colony PCR for rPX.

### Statistical Analysis

The data were presented as mean ± standard deviation, and Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) were used. According to the results of homogeneity of variance test, ANOVA was conducted for intergroup comparison, and least significant difference (LSD)-$t$ test was used as post-hoc test. $p < 0.05$ suggested that the difference was statistically significant.

## Results

### Construction and Identification f Recombinant Plasmids

The bacteria of 4 kinds of transfected recombinant plasmids (positive clone) were selected for colony PCR, of which the products underwent 1% agarose gel electrophoresis. The results (Figure 1) showed that the sizes of the electrophoresis bands were consistent with the predictions, indicating that the recombinant plasmids rCX, rEX1, rEX2 and rPX had been constructed successfully. Sequencing analysis was conducted for the inserted fragments and partial vector sequences in the recombinants, and the results revealed that the connection sites and reading frames were correct, and the detected sequences were identical to the known ones.

### Detection of HBx Messenger RNA (mRNA) Expressions and Expression Differences Among the Plasmid Transfection Groups via RT PCR

The RT-PCR electrophoresis results for HepG2/rCX, HepG2/rEX1, HepG2/rEX2, HepG2/rPX, HepG2/pGEM®-7Zf(+) and HepG2 cells are shown in Figure 2. No HBV X gene expressions were detected in HepG2/pGEM®-7Zf(+) control group and HepG2 blank group, while those expressions were detected in HepG2/rCX, HepG2/rEX1, HepG2/rEX2 and HepG2/rPX, of which the gray scale values of the transcription bands were (1.189±0.102), (0.877±0.043), (0.840±0.028) and (0.669±0.051), respectively; comparing the grayscale value of transfection group rCX with those of transfection groups rEX1, rEX2 and rPX, $p < 0.05$; comparing transfection groups rEX1 and rEX2 with transfection group rPX, $p < 0.05$; comparing the grayscale values between rEX1 and rEX2, $p > 0.05$. The relationships of the expression levels among the groups were HepG2/rCX->HepG2/rEX1 and HepG2//rEX2>HepG2/rPX.

### Detection of HBx Expressions via Western Blotting

It was found through Western blotting that among the transfection groups and the control group, the HBx expression was only detected in HepG2/rCX cells, and no corresponding X protein band was found after the total protein loadings of HepG2/rEX1 and HepG2/rPX cells were increased to the maximum. This might be caused by the low expression level; meanwhile, the X gene-mutated HepG2/rEX2 cells could not express X protein, and both the blank group and the control group did not contain X gene. Thus, X protein expression was not detected (Figure 3).

### Detection of Impact of HBx on Cell Proliferation via MTT Method

MTT method was applied to measure the $OD_{490}$ values of HepG2/rCX, HepG2/rEX1, HepG2/

**Table I.** Primer sequences.

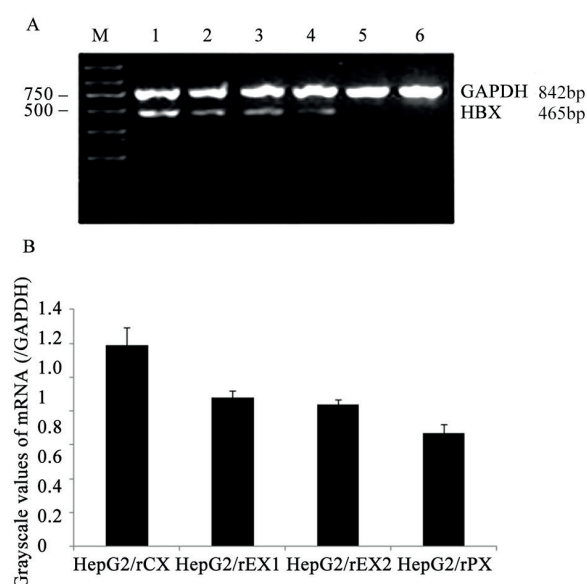| Gene | Primer sequence | Amplification product |
|------|-----------------|----------------------|
| HBx | Upstream: 5'-GCTCAAGCTTCCATGGCTGCTAGGCTGTGC-3' <br> Downstream: 5'-GGGGTACCTTAGGCAGAGGTGAAAAAGTTGC-3' | 465 bp |
| GAPDH | Upstream: 5'-GGA AGG TGA AGG TCG GAG TC-3' <br> Downstream: 5'-GAC CAC CTG GTG CTC AGT GT-3' | 842 bp |

**Figure 2.** **Detection of HBV X gene expressions via RT-PCR.** M: DL2000, 1: HepG2/rCX, 2: HepG2/rEX1, 3: HepG2/rEX2, 4: HepG2/rPX, 5: HepG2/pGEM®-7zf(+), 6: HepG2. There was no expression of HBX gene in control group HepG2/pGEM-7zf(+) cells and blank control HepG2 cells. HBX gene expression was observed in HepG2/rCX, HepG2/rEX1, HepG2/rEX2, and HepG2/rPX groups. Comparison of expression levels of HBX in different groups were: HepG2/rCX>HepG2/rEX1, HepG2//rEX2>HepG2/rPX.

rEX2, HepG2/rPX, HepG2/pGEM®-7zf (+) and HepG2 cells, which reflected the number of viable cells. The results indicated that $OD_{490}$ was increased along with the extension of time within 6 days. On the sixth day, the $OD_{490}$ values of the 6 kinds of cells were (0.741±0.013), (0.947±0.017), (1.292±0.095), (1.186±0.119), (1.313±0.098) and (1.415±0.121), respectively; *t*-test was performed for pairwise comparisons, and the results are shown below.

1) By comparing the $OD_{490}$ values of rCX, rEX1 and rPX transfection groups with that in pGEM®-7zf(+) transfection group, $p<0.05$;

2) Through pairwise comparison of $OD_{490}$ values among rCX, rEX1 and rPX transfection groups, $p<0.05$;

3) By comparing the $OD_{490}$ values between rEX2 and pGEM®-7zf (+) transfection groups, $p>0.05$;

4) By comparing the $OD_{490}$ value of pGEM®-7zf (+) transfection group with that of blank group HepG2, $p>0.05$.

Those results suggested that HBx could inhibit the growth of hepatocellular carcinoma cell HepG2, and the higher the HBx expression level
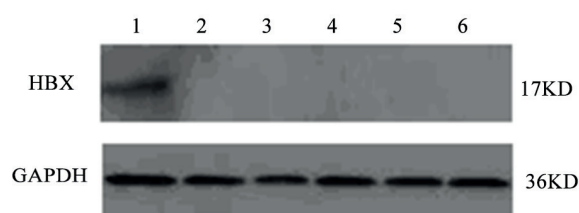


**Figure 3.** **Detection of HBx expressions via Western blotting.** 1. HepG2/rCX; 2. HepG2/rEX1; 3. HepG2/rEX2 4. HepG2/rPX; 5. HepG2/pGEM®-7zf (+); 6. HepG2. Expression of HBV X protein was detected in HepG2/rCX cells, but not in HepG2/rEX1, HepG2/rPX cells, HepG2/rEX2, blank group and control group.

was, the more significant the role of inhibiting cell growth would be (Figure 4).

## Detection of Impact of HBx on Cell Apoptosis via Flow Cytometry

In order to further determine the impact of HBx on cell apoptosis, the flow cytometry was conducted for HepG2/rCX, HepG2/rEX1, HepG2/rEX2, HepG2/rPX, HepG2/pGEM®-7zf (+) and HepG2 cells. It was indicated in the results that the cell apoptosis rates in those groups 48 hours after transfection were (22.497% ± 0.018%), (10.199% ± 0.010%), (5.211% ± 0.559%), (7.352% ± 0.733%), (5.107% ± 0.388%) and (4.611% ± 0.380%), respectively, by comparing the apoptosis rates of rCX, rEX1 and rPX transfection groups with that in
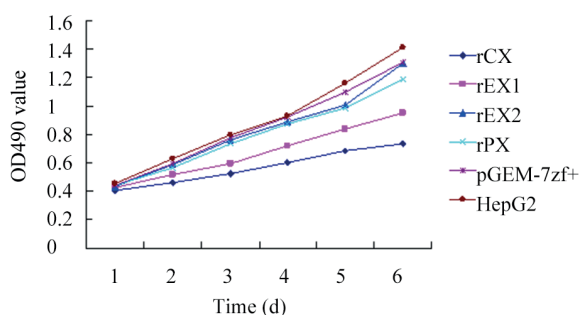


**Figure 4.** **Cell growth curves for HepG2/rCX, HepG2/rEX1, HepG2/rEX2, HepG2/rPX, HepG2/pGEM®-7zf(+) and HepG2.** HepG2/rCX, HepG2/rEX1, HepG2/rEX2, HepG2/rPX, HepG2/GEM-7zf (+) and control HepG2 cells were transfected into 96-well plates at 1x103 cells/well on the second day after transfection. Then, 10 ml MTT (5 mg/ml) were added into each well every day from day 1 to day 6. After cell culture at 37°C for another 4 hours, supernatant was carefully discarded, and 150 ml DMSO were added into each well. Absorbance was measured at 490 nm wavelength after shaking for 10 minutes. Results showed that HBx inhibited the growth of hepatoma HepG2 cells in an expression level-dependent manner.

pGEM®-7zf(+) transfection group, $p<0.05$; through pairwise comparison of apoptosis rates among rCX, rEX1 and rPX transfection groups, $p<0.05$; by comparing the apoptosis rates between rEX2 and pGEM®-7zf(+) transfection groups, $p>0.05$; by comparing the apoptosis rate of pGEM®-7zf(+) transfection group with that of HepG2 blank group,

$p>0.05$. The above results manifested that HBx could accelerate the occurrence of HepG2 apoptosis, and the terminated mutation of HBV X gene could offset the pro-apoptotic effect. Moreover, the higher the HBx expression level was, the more notable the pro-apoptotic effect would be (rCX>rEX1>rPX) (Figure 5).
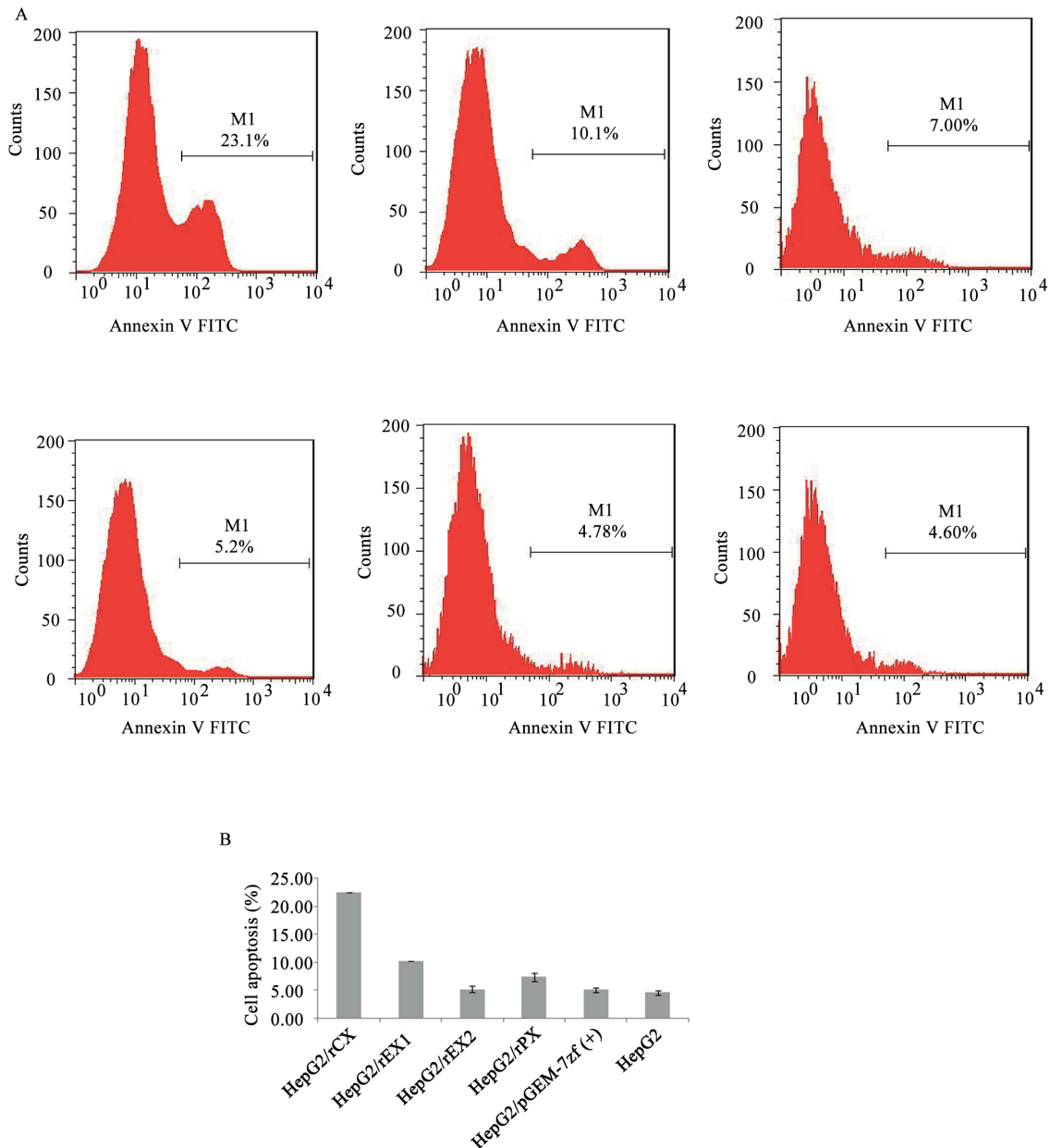


**Figure 5. Impact of HBx on cell apoptosis via flow cytometry.** From left to right in the top row: HepG2/rCX: 23.1%, HepG2/rEX1: 10.1% and HepG2/rPX: 7.0%; from left to right in the bottom row: HepG2/rEX2: 5.2%, HepG2/pGEM®-7zf(+): 4.8% and HepG2: 4.6%. HepG2 cells were transfected with 8 µg of recombinant plasmids rCX, rEX1, rEX2, rPX, and pGEM-7zf(+) vectors. 48 hours after transfection, each group of cells and control HepG2 cells were stained with Annexin-V/PI, and cell apoptosis rate of each cell was measured by flow cytometry. Results showed that HBV X protein promoted the apoptosis of HepG2 cells in a expression level-dependent manner.

## Discussion

The effects of HBx on cell apoptosis in different reports are not consistent; HBx can not only induce but also suppress the cell apoptosis, which may be determined by the cells it acts on [7]. Some authors[8] held that, in addition to the basis of experimental conditions and cell types, the HBx level in the cells can decide the direction of its regulation on apoptosis. Possible mechanisms of HBx-induced cell apoptosis include: (a) HBx can induce cells from G1 phase to S phase, and promote its apoptosis[9]; (b) HBx increases sensitivity of cells to p53-mediated apoptosis[10]; (c) HBx can upregulate the expression of Fas and FasL on the surface of hepatocytes, and promote hepatocyte apoptosis through the Fas/FasL death receptor pathway[11]; (d) HBx promotes TNFα-mediated cell apoptosis by activating MEKK1/JNK signal transduction pathway and promoting the accumulation of oncoprotein myc in the nucleus, therefore increasing the sensitivity of cells to TNFα; (e) in the early stage of apoptosis, HBx is overexpressed; Ca2+ in the cytoplasm is increased; mitochondrion structure and function are changed; PT channel is opened; cytochrome oxidase C and other proapoptotic factors are released, and caspase cascade reaction is activated, which in turn induces apoptosis [12]. In this research, the recombinant vectors in which the expressions of HBV X gene were regulated by different promoters were constructed, so as to study the difference in the impacts of hepatocellular carcinoma cell line HepG2 transiently transfected by them on cell apoptosis, and to investigate the role of HBx in the pathogenic mechanisms of HBV and hepatocellular carcinoma. The results of this research indicated that the expressions of HBV X gene could be detected in the HepG2/rCX, HepG2/rEX1, HepG2/rPX and HepG2/rEX2 transfection groups via RT-PCR, which conforms to the results of most studies at present. However, the Western blotting results indicated that the HBx expression was only detected in the HepG2/rCX transfection group; the reason might be that the HBx expression levels in the HepG2/rEX1 and HepG2/rPX transfection groups were so low that they could not be detected; in transfection group HepG2/ rEX2, terminated mutation occurred in the eighth codon of the HBV X gene; as a result, the HBx expression was not detected. Four types of transcription products of HBV (including HBx) are regulated by two enhancers, namely Enh1 and Enh2; Enh1 is embedded in P gene, whose downstream part is overlapped with X promoter, thereby enhancing the X, C and S promoters; Enh2 is located in X gene. Su et al[13] have reported that the two enhancers can strengthen the activity of HBV promoter in many cell lines, and the enhancers exert a synergistic effect. HBx expression is mainly regulated by Enh1; it is mainly manifested that Enh1 can increase the transcriptional activity of X promoter by combining with multiple tissue-specific transcription factors; therefore, the extrinsic factors that can strengthen the transcription factor expressions can also indirectly increase the HBx expression by virtue of Enh1. For example, insulin and interleukin-6 (IL-6) can elevate the X gene expression by increasing that of the transcription factor, activator protein-1 (AP-1)[14,15]. Tumor necrosis factor can promote the HBx expression by stimulating the generation of free radicals, thus enhancing the transactivating functions of HBx[16,17], and IL-6 can improve the HBx expression by means of stimulating HBV Enh1 and X gene promoter[18]. In this research, the HBx expression was detected only when HBx was regulated by the strong promoter CMV, but it was not detected under the regulation of its X promoter and (or) Enh1, for which the possible reason was the low HBx expression level due to lack of stimulation of tumor necrosis factor and IL-6 during experiments *in vitro*. In further experiments, the HBx expression level can be elevated by adding stimulating factors that can regulate it (including tumor necrosis factor) into corresponding cell culture. These stimulating factors may regulate the HBx expression through regulation of HBV enhancer and promoter activities, thereby controlling the effect of HBV on cell proliferation or apoptosis. It could be observed from this experiment that HBx played a role in promoting cell apoptosis in both low- and high-expression states; when the terminated mutation of the eighth codon of HBV X gene was triggered, the pro-apoptotic effect of the gene also disappeared. Moreover, the pro-apoptotic effect of HBx was related to its expression level, and the higher the HBx expression level was, the more notable the pro-apoptotic effect would be. For some of the patients with liver failure, the cause of the disease is the mass necrosis of hepatocytes resulted from massive replication of HBV DNA; HBx may have a certain function in the mechanism besides the immune factors because HBV replication has a positive correlation with HBx expression[19,20]. Correspondingly, the HBx is also highly expressed during the massive replication of HBV, and its pro-apoptotic effect is becoming evident, thereby leading

to apoptosis of more cells and playing a certain role in the progression of liver failure. However, the regulation of apoptosis by HBX is bidirectional, whether blocking or inducing apoptosis depends on the cell type, the living environment of the cell and the stage of infection. In this work, only one hepatoma cell line HepG2 was used, and other types of cells should be used to analyze the mechanism of apoptosis and proliferation regulated by HBx, in which case the effects of TNF or IL-6 stimulation on HBx expression, cell proliferation and apoptosis were observed.

## Conclusions

HBx expression is at a low level under the regulation of self-promoters and HBV enhancers; the exogenous pathogenic factors for liver injury may regulate the HBx expression by controlling the activities of HBV enhancers and promoters, so as to regulate the impact of HBV on cell apoptosis. It is further indicated that the pathogenesis of HBV-induced hepatocellular carcinoma is complex; in addition to the role of the virus, various pathogenic factors causing pathological changes of hepatocytes may play a role in the occurrence of hepatocellular carcinoma. As a result, reducing relevant risk factors of liver injury to keep the HBx expression at a correspondingly low level may be significant in lowering the occurrence of hepatocellular carcinoma.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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